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MODERN APPLICATIONS OF ANALYTICAL ULTRACENTRIFUGATION

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ABSTRACT

Analytical ultracentrifugation is a classical method of biochemistry and molecular biology. Because it is a primary technique, sedimentation can provide first-principle hydrodynamic and first-principle thermodynamic information for nearly any molecule, in a wide range of solvents and over a wide range of solute concentrations. For many questions, it is the technique of choice. This review stresses what information is available from analytical ultracentrifugation and how that information is being extracted and used in contemporary applications.

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INTRODUCTION

Analytical ultracentrifugation is a classical method of biochemistry and molecular biology. Because analytical ultracentrifugation relies on the principal property of mass and the fundamental laws of gravitation, it has broad applicability. It is a primary technique requiring no standards for comparison. Sedimentation can be used to analyze the solution behavior of nearly any molecule over a wide range of solute concentrations and in a wide variety of solvents. Thus, while low concentration regimes are of interest for analyzing tight associations, the ability to characterize the thermodynamic behavior of a macromolecule at high concentrations makes ultracentrifugation a good adjunct for drug formulation studies, NMR, or crystallography. Add to these merits the facts that sedimentation is nondestructive, rapid, and simple, and it is easy to see why it has endured for more than 70 years.

Analytical ultracentrifugation provides two complementary views of solution behavior (Figure 1). Although the same instrument is used, different experimental protocols are employed. Sedimentation velocity provides first-principle, hydrodynamic information about the size and shape of a molecule, whereas sedimentation equilibrium provides first-principle, thermodynamic information about the solution molar mass, association constants, stoichiometries, and solution nonideality. For many questions, there is no substitute method of analysis.

History

For newcomers to the field, it is important to realize that the early literature is relevant. A history of analytical ultracentrifugation is available (135), and excellent books and reviews by Svedberg (131), Schachman (115), Williams

Figure 1 Two complementary methods are available using an analytical ultracentrifuge. Interference images are shown in which the vertical displacement of fringes is proportional to the concentration at each radial position. For sedimentation velocity (*A*) a high rotor speed and a long solution column is used to maximize the resolution of species. The sedimentation coefficient, s, is determined from the rate of boundary movement, whereas the rate of boundary spreading is used to determine the diffusion coefficient, D. These two quantities describe the hydrodynamics of a molecule, while their ratio, s/D, can be used to determine its buoyant mass. For mixtures of molecules, it is possible to determine M_b for each species forming a discrete boundary. Sedimentation equilibrium analysis (*B*) uses lower rotor speeds and shorter solution columns. At equilibrium, the flux of sedimenting molecules is exactly balanced by the flux of diffusing molecules at each radial position. Analysis of the resulting concentration distribution provides insight into the thermodynamic behavior of the sedimenting species. Solution molar masses, association constants, association stoichiometries, and nonideality coefficients may be determined.

(141, 142), Cassasa & Eisenberg (14), and Fujita (33) cover the invariant foundations of ultracentrifugation. More recent books (44, 85, 102, 119) and reviews (37, 38) cover many of the improvements made in the past decade.

This review will stress what information is available from analytical ultracentrifugation using the Beckman-Coulter XLI equipped with absorbance and interference optical detectors (65). In so doing, it will neglect techniques that use preparative centrifuges and post-sedimentation analysis (5, 6, 22, 54, 87, 107). Also, density gradient and sucrose gradient sedimentation are not covered (81, 82, 88, 106, 129). Finally, there is a vast literature on the methods of analysis of sedimentation data. Space limitations prevent a comprehensive review of each technique. Instead, references are made to the pertinent literature.

GENERAL FEATURES

Sedimentation velocity and sedimentation equilibrium share several features. For both methods, the fundamental measurement is the concentration as a function of radial position. Interpretation of these data requires knowledge of the rotor speed, temperature, elapsed time, viscosity, and buoyancy.

Concentration Distribution

The concentration distribution is required for analysis of sedimentation data. Issues surrounding the measurement of the concentration distribution include the precision, accuracy, linearity, range, sensitivity, and selectivity with which it can be determined. Both absorbance and interference detectors are commonly used (65).

Absorbance detection provides greater selectivity and, depending on the characteristics of the solute and solvent, superior sensitivity. Obviously, use of absorbance detection is limited to samples that absorb light and buffers that do not. For the XLI, the linearity of the absorbance system is best below about 1.5 OD. This means that the concentration range may be limited, though this often can be overcome by combining data acquired at different wavelengths.

Interference detection provides a signal that is proportional to the difference in refractive index between a sample and a reference solution. This optical system provides superior linearity, range, and accuracy compared to the absorbance system, but it offers no selectivity. There is no need for a chromophore, which makes it useful for working with nonabsorbing materials such as polysaccharides. The sensitivity of interference detection is roughly comparable to that of the absorbance system for proteins at 280 nm (65). The linearity is limited only by the quality of the optics, and accurate data can be obtained at very high concentrations (150).

Recently, fluorescence/luminescence optics have been described for the XLI (66). While the range, linearity, and precision are still being characterized, it appears that the superior sensitivity and selectivity of the fluorescence detection will be useful.

Rotor Speed and Temperature

Rotor speed is expressed as the angular velocity, ω, in cgs units, where $\omega = \frac{\text{rpm} \cdot \pi}{30}$. The rotor speed is readily determined from the period of rotation, and is maintained with great accuracy (71).

The temperature must be known and stable. This is especially true for velocity sedimentation because of the large temperature dependence of viscosity. Two sensors provide temperature readings for the XLI, with a radiometer being used at chamber pressures below 100 μm. Temperature is regulated by a proportional control, which provides an accuracy and stability better than $\pm 0.1^{\circ}$C. The

accuracy of this system can be checked using the absorbance optics and solutions of ethanolic cobalt chloride (55, 128).

Elapsed Time

Two different elapsed times are pertinent in analytical ultracentrifugation. The first is the reduced sedimentation time, $\int \omega^2 t$, which is needed for determining the sedimentation coefficient. The second is the time from the start of the experiment, t, which is needed to determine the diffusion coefficient. The XLI maintains both t and $\int \omega^2 t$ (as $\sum \omega^2 \Delta t$) updated at 1-second intervals. These values are calculated and stored by the centrifuge hardware and accessed by the software from the external computer.

Viscosity

Viscosity is a solution property that inversely affects sedimentation velocity (148). The viscosity of an aqueous solution depends strongly on its temperature and the concentration of macromolecules, and more weakly on the concentration of salts and other small solutes. Observed sedimentation coefficients, s^*, must be corrected to the standard conditions of pure water at 20°C (below). For aqueous solutions containing low concentrations ($\lesssim$0.2 M) of salts, the concentration corrections are relatively minor; the temperature correction usually is taken to be the same as that for pure water (69). The solvent viscosity does not affect the final concentration distribution in sedimentation equilibrium, but does affect the time needed to reach equilibrium directly.

Buoyancy

Archimedes' buoyancy principle applies at the molecular level. Consequently, the apparent net mass of a particle in solution is the anhydrous particle mass less the mass of the solvent it displaces. If M is the solute molar mass (expressed in g/mole), the displaced solvent mass is $M\bar{v}\rho$, where $\bar{v}$ is the solute's partial specific volume (in ml/g) and ρ is the *solvent* density (in g/ml) (134). It is the net, or buoyant, mass, $M_b = M(1 - \bar{v}\rho)$, that always appears in sedimentation equations.

To a first approximation, $\bar{v}$ is the reciprocal of the density of the solute. The packing of solvent molecules around a particle also influences $\bar{v}$. For example, a charged particle will compact polar solvent in its vicinity, leading to a smaller value of $\bar{v}$ than otherwise might be anticipated (84). Partial specific volume can be measured from the density increment (28–30, 133) or from density perturbation sedimentation equilibrium (31) or can be calculated from the composition (32, 69, 84, 97).

Any error in the buoyancy term propagates as a threefold error in M. Hence, accurate values of $\bar{v}$ and ρ are needed to calculate M from sedimentation data. It is relatively simple to measure or calculate ρ to within a few tenths of a percent

error, and $\bar{v}$ typically can be estimated to within 1%. This means that for a pure sample exhibiting ideal solution behavior, M can be determined to within 3% without difficulty.

Advantage can be taken of the fact that $\bar{v}$ is primarily a property of a solute, whereas ρ is a property solely of the solvent. Through appropriate adjustment of ρ with D_2O or $D_2{}^{18}O$ (i.e. to make $1-\bar{v}\rho = 0$), a molecule can be made neutrally buoyant (i.e. it neither sediments nor floats), so that it does not contribute to the buoyant mass of other macromolecules to which it binds. This property can be of tremendous use when examining proteins in detergent-containing solvents. (86, 104, 105, 111, 112, 132, 139).

SEDIMENTATION VELOCITY

Sedimentation of a Single Species

While a rigorous derivation of the sedimentation velocity equation can be made using the framework of nonequilibrium thermodynamics (134), a more intuitive approach considers the balance of forces on a particle in a gravitational field. When the gravitational field is first applied, the particles come to a constant velocity almost instantaneously. Since the velocity is constant, there can be no net force, meaning the gravitational and frictional forces are exactly balanced.

The gravitational force is $M_b\omega^2 r$, where r is the distance from the center of rotation (in cm). Both ω^2 and r are determined automatically and with great accuracy by the analytical ultracentrifuge. The frictional force is directly proportional to the particle's Stokes radius, R_s and velocity, dr/dt, as well as to the solution viscosity, η. The frictional force is written as $f\, dr/dt$, where f is the translational frictional coefficient. The interpretation of f is discussed below. With few exceptions (21), there is no alignment of particles in the gravitational field, so f is a property of the entire molecule.

The balance of forces leads to $M_b\omega^2 r = f\, dr/dt$, which is rearranged to give the two definitions of the apparent sedimentation coefficient, s^*:

$$s^* \equiv \frac{\text{velocity}}{\text{acceleration}} = \frac{\frac{dr}{dt}}{\omega^2 r} = \frac{d\ln r}{\omega^2\, dt} = \frac{M_b}{f} \qquad 1.$$

The first definition, $d\ln r/\omega^2\, dt$, describes the experimental observations needed for the measurement of s^*, whereas the second definition, M_b/f, involves the properties of the particle and solvent. Since the buoyant mass (for incompressible solvents) and the frictional coefficients are constant, s^* is a constant. The sedimentation coefficient contains both thermodynamic information, through M_b, and hydrodynamic information, through f. An independent determination of either M or f must be available to interpret s^* further.

The velocity of a boundary (Figure 2) can be determined as the distance moved from the meniscus divided by the reduced sedimentation time. Thus, it is possible to determine s^* from:

$$s^* = \frac{\ln \frac{r}{r_m}}{\int \omega^2 \, dt}, \qquad 2.$$

where r_m is the meniscus position. For any given time, Equation 2 can be used to transform the radial axis into an s^* axis. This transformation is used by many of the modern analysis methods (below).

Diffusion

Diffusion causes the boundary to spread as it moves down the cell (Figure 2). Since there is no net force on the molecules during sedimentation, boundary spreading is independent of sedimentation, and is described by Fick's first law. The diffusive flux, J_D, of material at a point, r, in the concentration boundary obeys:

$$J_D = -D\left(\frac{\partial c}{\partial r}\right)_t \qquad 3.$$

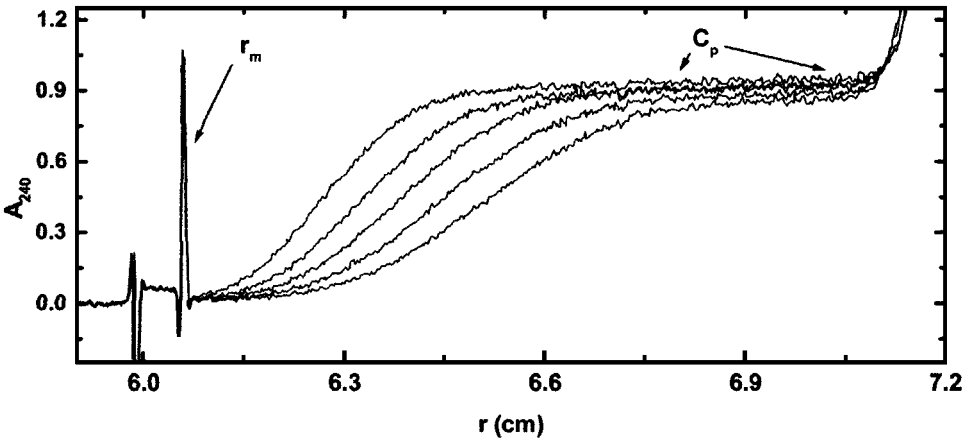

Figure 2 Absorbance distributions at 240 nm acquired at 20-min intervals during a sedimentation velocity experiment with porcine pancreatic ribonuclease (~2 mg/ml) at 20°C and 60,000 rpm in 100 mM KCl, 10 mM BisTrisPropane, pH 6.5. The meniscus position (r_m) is marked, as is the region where the plateau concentration (C_p) can be measured. The plateau concentration decreases with time due to the sector shape of the cell. The sector shape is required to keep the walls parallel to the radially directed gravitational force.

where c is the concentration. Several analysis methods provide estimates of the translational diffusion coefficient, D, in addition to determining s^*. This is of great use because of the Einstein-Boltzmann-Sutherland relationship between D and f:

$$D = \frac{k_B T}{f}, \qquad 4.$$

where k_B is Boltzmann's constant and T is the absolute temperature. It must be stressed that this interpretation of boundary spreading is valid only for dilute solutions. At higher concentrations, both thermodynamic and hydrodynamic nonideality may distort the boundary shape.

Hydrodynamic Nonideality

Hydrodynamic nonideality results from the counterflow of solvent from a sedimenting particle slowing the motion of a neighboring particle (41–43, 95, 113). Since the solvent flow is in the direction opposite to sedimentation, s^* is always decreased by hydrodynamic nonideality. It can be shown that a graph of $1/s$ versus c, over a wide range of concentrations, yields a straight line with slope k_s. If the charge effects between particles are small, k_s can provide a sensitive measure of molecular asymmetry:

$$k_s = 2\bar{v}\left\{\frac{v_s}{\bar{v}} + \left[\frac{f}{f_o}\right]^3\right\}, \qquad 5.$$

where v_s is the specific volume of the solvated particle and f_o is the minimum frictional coefficient (69). There is no adequate interpretation for k_s if charge effects are significant. However, for an uncharged sphere, k_s is $\sim$0.004 ml/mg. This value increases by almost two orders of magnitude for asymmetric molecules (40–43, 113). Since s^* can easily be measured with a precision of 1–2%, hydrodynamic nonideality will not be significant for spherical molecules at concentrations $\leq\sim$5 mg/ml. However, for more asymmetric molecules, the effects can be apparent at concentrations below 0.1 mg/ml (42, 43). For a noninteracting system, it is best to extrapolate values of s^* to $c = 0$, yielding s^o.

Hydrodynamic nonideality also influences the shape of a sedimenting boundary. However, both thermodynamic and hydrodynamic nonideality influence boundary spreading, with the former increasing the rate of spreading, while the latter sharpens a boundary. Thus, for an accurate determination of D, it is important to extrapolate measurements to $c = 0$, yielding D^o.

Correction of s^o and D^o to Standard Conditions

Both s^o and D^o are dependent on properties of both the solute and of the solvent. It is desirable to have s and D in a form that makes them dependent only

on the solute properties (i.e. M_b and f), so that values acquired at different temperatures or in different solvents may be compared and discussed in terms of changes to the solute. To account for the solvent properties, s^o is adjusted for the solvent effects on buoyancy (through ρ and $\bar{v}$), and both s^o and D^o are adjusted for the solvent effects on f (through the viscosity, η). The standard conditions used for sedimentation are those of pure water at 20°C. The values of s^o and D^o at standard conditions are calculated as

$$s^o_{20,w} = s^o \left(\frac{(1 - \bar{v}\rho)_{T,b}}{(1 - \bar{v}\rho)_{20,w}} \right) \left(\frac{\eta_{20,w}}{\eta_{T,b}} \right)$$
$$D^o_{20,w} = D^o \left(\frac{\eta_{20,w}}{\eta_{T,b}} \right), \qquad 6.$$

where values for water at 20°C are denoted by subscripts 20,w, and values for the solvent at the experimental temperature are denoted by subscripts T,b (28, 69).

Determining M_b

One of the earliest uses for analytical ultracentrifugation was the determination of the molecular weight of dissolved macromolecules using the Svedberg equation:

$$\frac{s}{D} = \frac{\frac{M_b}{f}}{\frac{RT}{f}} = \frac{M_b}{RT}, \qquad 7.$$

where R is the cgs gas constant (8.314×10^7 erg/mole-°K). Notice that s and D do not have to be adjusted to standard conditions to determine M_b; however, the accuracy of this method does depend on both thermodynamic and hydrodynamic ideality holding. Also, ρ and $\bar{v}$ need to be determined for the experimental conditions to calculate M from M_b. Because it is not difficult to analyze globular proteins under conditions where ideality holds, sedimentation velocity provides a rapid and simple means of determining solution molecular weights with a precision of about 5%.

Interpretation of $s^o_{20,w}$

If M_b is known, it is possible to interpret $s^o_{20,w}$ in terms of the frictional coefficient of the solute. The frictional coefficient, in turn, is dependent on the Stokes radius of a particle, R_s, and the solvent viscosity: $f = 6\pi\eta R_S$. Both the solvation and the shape of the particle affect R_s. The solvation is important because the sedimenting particle drags a shell of solvent with it, thus increasing its effective size. In aqueous solutions, the concept of solvation is replaced by

that of hydration, which usually falls into the range of 0.3–0.4 g-H_2O/g-solute (69).

The shape of the particle also affects f, with spherical particles having the smallest possible friction for a given mass. The effect of shape on $s^o_{20,w}$ is interpreted using one of two approaches. The first, referred to as the whole-body approach, expresses f in terms of generalized ellipsoids of revolution and is based on the pioneering work of Perrin (39, 40). This method yields the principal axes of an ellipsoid as a structural description of the sedimenting particle. The second approach is an extension of the Kirkwood-Bloomfield analysis in which a structure is represented by beads arranged in space (13, 23, 24). The intrinsic frictional force of each bead is adjusted by the velocity of the local solvent, which is being dragged in the same direction by adjacent beads. These forces are summed to give the frictional force on the whole particle. Ordinarily this method is used for solutes for which there is already good structural information (e.g. for a subunit) when questions are being asked about the solution conformation (92). Both methods use models to try to extract multiple parameters from a single measurement, f. Thus, both are subject to error if underlying assumptions are not met.

Multiple Components

In the simplest case of a mixture of noninteracting components, a separate boundary will form for each component in solution. If well-resolved, and under conditions where hydrodynamic and thermodynamic ideality hold, each boundary can be analyzed separately to get estimates of M_b and f. This method of analysis has found recent use in the analysis of the stoichiometry and the shape of antigen-antibody complexes where the assemblies are sufficiently stable to be considered distinct species (76).

Sedimenting boundaries often overlap. Sometimes it is possible to resolve them using time-derivative methods and Gaussian deconvolution (125, 127). More often, though, overlapping boundaries cannot be resolved. In such cases, a well-defined average sedimentation coefficient can still be determined. Consider the rate at which the plateau region (Figure 2) is depleted. This rate reflects the concentrations, c_i, and sedimentation coefficients, s_i, of all species in a mixture. The weight-average sedimentation coefficient, $\bar{s}_w$, is

$$\bar{s}_w = \frac{\sum_i s_i c_i}{\sum_i c_i}. \qquad 8.$$

It can be shown (142) that the equivalent information is available from monitoring the rate of movement of the second moment of the concentration distribution.

The determination of the second moment position has been largely automated, so $\overline{s}_w$ is easily determined.

The weight-average D is not available from a sedimentation velocity experiment. Instead, the gradient-average, $\overline{D}_g$, is obtained at the second moment position. The ratio of $\overline{s}_w/\overline{D}_g$ does not yield a well-defined average molecular weight, because the averages $\overline{s}_w$ and $\overline{D}_g$ are of a different nature.

There are hydrodynamic consequences of having multiple species present. One is the Ogston-Johnston effect, which arises because the faster species in a mixture sediment in a solution containing the slower moving species, whereas the slowest moving species sediments in pure solvent (58, 95). This effect not only results in inaccuracies when determining the sedimentation coefficients, but also can adversely affect the accuracy of concentration determinations of the different components.

Interacting Systems

The sedimentation velocity behavior of an interacting system is more complicated than that of an equivalent mixture of noninteracting components. The complexity arises from the coupling of transport with the mass-action re-equilibration of the transiently formed complexes (11). Consequences include boundaries whose shapes vary with total concentration, and values of $\overline{s}_w$ that, in the absence of hydrodynamic nonideality, increase with increasing concentration (62, 77–79). The rate of re-equilibration relative to the rate of transport determines the boundary shape (11). The two limiting cases, instantaneously fast or infinitely slow re-equilibration, give rise to boundary shapes that are independent of rotor speed. The intermediate case of kinetically limited re-equilibration results in boundary shapes that depend on the sedimentation rate. Infinitely slow re-equilibration is observed if the rate of dissociation occurs on a time scale of 30 minutes or greater. Instantaneously fast re-equilibration will be observed if disassociation rates occur on a time scale of $\sim$0.1 second or less. All cases may be analyzed profitably, though, if the system is at equilibrium at the start of an experiment (11, 126). If the kinetics are not infinitely slow, the boundaries observed in a re-equilibrating system do not correspond to individual species. Instead, they always consist of a mixture of species, and are called reaction boundaries. The only possible exception to this is the slowest moving boundary, which may correspond to the smallest species (11).

Frequently, the kinetics and thermodynamics of an interacting system are influenced by the binding of small ligands. In such cases, the shape of the reaction boundaries may be influenced by the local concentration of the ligand (11, 12), particularly if the free ligand concentration is depleted significantly by the sedimenting macromolecule (140). In such cases, valuable information

is available from the analysis of experiments conducted at varying ligand concentrations.

Methods of Analysis

Because of the wealth of information available on multicomponent and interacting systems, the analysis of sedimentation velocity experiments is an active area of research (7, 18, 25, 47, 118, 136). Demeler has largely automated the production of graphs that provide diagnostics for ideal sedimentation, hydrodynamic nonideality, thermodynamic nonideality, and interacting systems (25, 136). Detailed analyses for s and D are available from curve-fitting methods based on solutions of the Lamm equation (7, 47–49, 98, 117). The recent development of a time-derivative method, resulting in vastly improved signal-to-noise ratios, also allows for determination of s and D from complex mixtures (125) and for the analysis of interacting systems (126). Finally, fast computers and efficient algorithms have permitted the use of finite-element simulations as fitting functions (117). This opens the way for direct fitting to the transport equations for interacting systems and ligand-linked interacting systems (127). A recent review of these methods is available (18).

SEDIMENTATION EQUILIBRIUM

The defining characteristic of sedimentation equilibrium is the time-invariant concentration gradient that develops as the flux of sedimenting molecules is exactly balanced by the flux of diffusing molecules at each point in the cell. No distinct boundaries are observed; instead a smooth gradient is seen (Figures 1 and 3). Hydrodynamics affect the length of time needed to reach equilibrium, but only thermodynamics affect the equilibrium concentration gradient.

The equations that describe sedimentation equilibrium may be derived from a number of approaches (14, 33, 35, 134, 142). Two approaches are used in this review. The first is based on the balance of fluxes and is used when discussing the processes that contribute to equilibrium. The second is based on the balance of energies and is used when discussing the thermodynamics of an equilibrium distribution.

Processes Leading to Equilibrium

For a single, thermodynamically ideal component, an equation describing sedimentation equilibrium is obtained from an analysis of the flux of material, in moles per second per cm^2, passing through a plane, P, of area A (Figure 3). The flux through P due to sedimentation, J_s, is the concentration of macromolecules times their velocity: $J_s = \frac{c \cdot v}{A} = \frac{c \cdot s\omega^2 r}{A}$, where the velocity, v, is defined in terms of the sedimentation coefficient (Equation 1). The flux through P due to

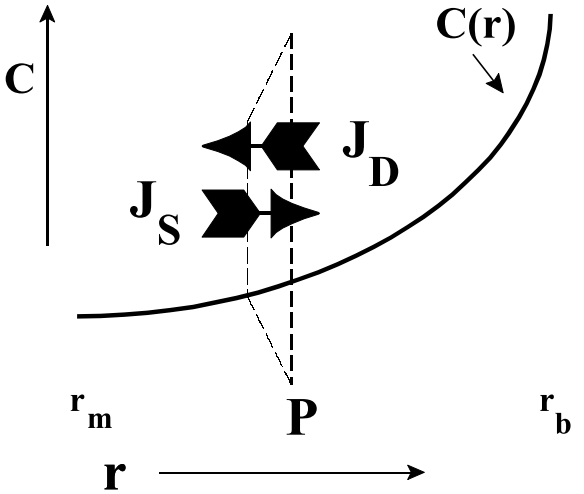

Figure 3 A schematic of the process of sedimentation equilibrium. A solution containing macromolecules is confined to the region between the air-liquid meniscus (r_m) and the base of the cell (r_b). The gravitational field points from left to right and is directed along the radial axis (r). At any point on this axis, the gravitational field is equal to $\omega^2 r$.

diffusion is described by Fick's first law (Equation 3). At equilibrium, these two fluxes are equal, so that $c\,s\omega^2 r = D\frac{dc}{dr}$. Collecting terms, this becomes

$$\frac{s\omega^2}{D} = \frac{1}{rc}\frac{dc}{dr} = \frac{d\ln c}{d\frac{r^2}{2}} \equiv \sigma$$

or

$$\sigma \equiv \frac{M_b\omega^2}{RT}, \qquad 9.$$

where the first definition of σ is in terms of the experimental variables c and r, and the second definition is in terms of the molecular weight. The sensitivity of σ to rotor speed makes sedimentation equilibrium useful over a wide range of molecular weights.

Integration of Equation 9 yields

$$c(r) = c_o e^{\sigma\frac{r^2-r_o^2}{2}} = c_o e^{\sigma\xi}, \qquad 10.$$

where c_o is the concentration at a reference radius, r_o, and σ is the exponent (153). This simple equation is the starting point for understanding more complex systems.

Generally, a sedimentation equilibrium experiment is conducted at rotor speeds that result in values of σ between 2 and 15 cm^{-2}. Under these circumstances, and for a typical cell height of 3 mm, the concentration at the base of the cell is about 2 to 10 times the concentration at the meniscus (150). Generally, several dilutions of a sample are examined simultaneously, and at different rotor speeds, yielding several thousand data points spanning a 100-fold or greater concentration range. The availability of data from such a wide concentration range makes it possible to accurately describe the solution behavior of a molecule.

Time to Reach Equilibrium

The exact length of time needed to establish sedimentation equilibrium is a complicated function of the experimental parameters and cannot be accurately predicted except in the simplest cases (8, 20, 83, 149, 150). Although attainment of equilibrium is determined empirically, the approximate time needed follows a few simple rules. In general, repulsive thermodynamic nonideality will shorten the time needed to achieve equilibrium. On the other hand, the equilibration time increases as the square of the distance from the meniscus to the base of a solution column. Thus, the use of shorter solution columns will speed up equilibrium considerably, although with loss of precision (20, 63, 149). The time needed to reach equilibrium also increases proportionally with the viscosity of the sample. Furthermore, kinetic processes, such as mass-action associations, may increase the time needed to reach equilibrium. In extreme cases, slow kinetic processes may prevent equilibrium from being established in a reasonable time period. This is discussed further below.

An empirical demonstration of equilibrium is often made by looking for a zero concentration difference when two concentration profiles, taken an hour or so apart, are subtracted (150). A better procedure is to monitor the minimized average differences in concentration profiles over time, such as is done by MATCH (David Yphantis and Jeff Lary, available from ftp://rasmb.bbri.org). The results from MATCH also indicate the intrinsic noise level of the data. This is useful information when analyzing data using a nonlinear least-squares approach.

Ideal Solutions

Goldberg showed that sedimentation equilibrium results in a thermodynamic system in which the total internal energy (E) is minimized at each position in the cell (33, 35). At each point the gravitational potential energy of a particle, $-\frac{1}{2}M_b\omega^2r^2$, is exactly balanced by its electrochemical potential energy, μ. The energetic contributions to sedimentation equilibrium can be understood from consideration of this balance. For example, the gravitational potential will

vary if M_b varies with pressure (that is, with r). This would be expected if a solvent is compressible, leading to an increasing density with r and therefore a decreasing M_b with r (34). Similarly, pressure-dependent changes in $\bar{v}$ will affect the gravitational potential through its influence on M_b.

Fortunately, M_b is constant for biological molecules in aqueous solutions. Thus, the concentration gradient that develops can be analyzed in terms of the electrochemical potential of the solute. There are several contributions to the electrochemical potential, including the inevitable concentration gradients in the solvent components (14), the Donnan potentials that result from the differences in sedimentation of a macromolecule and its counterions (9, 10, 19, 26, 110, 150, 152, 153), and the concentration dependence of the chemical potential of the macromolecule. Because it is this latter information that is usually sought in a sedimentation equilibrium experiment, it is necessary to minimize the first two contributions.

If a solute is brought to dialysis equilibrium with the solvent (i.e. the solution is made isopotential with respect to the solvent components), the solvent can be treated as a single component so long as the apparent isopotential partial specific volume, ϕ, of the solute is known in that solvent (14). Under these conditions, only the residual Donnan contributions must be considered, and these effects are usually small so long as solvents of sufficient ionic strength are used (10, 110). Fujita recently has extended this analysis to include compressible solvents (34).

To be useful, though, the relationship between ϕ and $\bar{v}$ must be defined. For proteins in dilute aqueous solvents, the variation in ϕ with solvent composition is small, and $\bar{v}$ may be used in its place. However, $\bar{v}$ will differ significantly from ϕ in aqueous solvents that contain high concentrations of a component (e.g. 8-M urea) or that contain a component that binds to the protein in significant levels (e.g. detergents). Methods have been developed to calculate ϕ for proteins in 6-M guanidinium chloride, 8-M urea, and certain other solvents (4, 59, 69, 72, 100, 101). Likewise, there are data and methods available for estimating ϕ in detergent-containing solutions (31, 104, 105, 130, 132). For polyelectrolytes such as DNA, ϕ can vary significantly with solvent composition and should be measured (28).

With these caveats removed, the concentration distribution observed in a sedimentation equilibrium experiment is a direct reflection of the chemical potential of the solute. However, it is the chemical potential related to the total internal energy, E, that is observed, and not the chemical potential related to the free energy, G. So long as a system is incompressible, the difference between these two is insignificant. Hence, the energetics determined by sedimentation equilibrium are usually reported as ΔG. Although the use of ΔG is appropriate for reactions involving only small volume changes in aqueous solutions, it

will not be valid if there are pressure-dependent effects. Fortunately, pressure dependence can be diagnosed and analyzed (45, 53, 60–62).

For a solution containing multiple ideal species, what usually can be measured is not the concentration of individual species, but rather the total concentration of all species in the solution:

$$c(r) = \sum c_i(r) = \sum c_{oi} e^{\sigma_i \xi}. \qquad 11.$$

Ideal, Reversible Self-Associations

The equation describing reversible self-association in an otherwise ideal solution is obtained by expanding Equation 11 in terms of the species:

$$c(r) = \sum c_{oi} e^{\sigma_i \xi} = c_{o1} e^{\sigma_1 \xi} + c_{o2} e^{\sigma_2 \xi} + c_{o3} e^{\sigma_3 \xi} + \cdots, \qquad 12.$$

where c_{o1} and σ_1 refer to reference concentration and reduced buoyant molecular weight of the monomer, c_{o2} and σ_2 refer to these quantities for the dimer, and so forth. For a reversible mass action association, all σ's are related, so that $\sigma_2 = 2\sigma_1, \sigma_3 = 3\sigma_1$, etc, so long as $\bar{v}$ for each species is the same. While this is generally a safe assumption, for cases where $\bar{v}$ is not the same, the relationships will be nonintegral. The monomer reference concentration, c_{o1}, is linked to the other reference concentrations, c_{on}, through the equilibrium constant

$$K_{a1 \Leftrightarrow n} = \frac{[c_{on}]}{[c_{o1}]^n}, \qquad 13.$$

where $[c_n]$ is the concentration of oligomer and $[c_1]$ is the monomer concentration. Substituting Equation 13 into Equation 12:

$$\begin{aligned} c(r) &= \sum c_i(r) = \sum c_{oi}^i K_{a1 \Leftrightarrow i} e^{i\sigma_i \xi} \\ &= c_{o1} e^{\sigma_1 \xi} + c_{o1}^2 K_{a1 \Leftrightarrow 2} e^{2\sigma_1 \xi} + c_{o1}^3 K_{a1 \Leftrightarrow 3} e^{3\sigma_1 \xi} + \cdots. \end{aligned} \qquad 14.$$

Heterogeneous Mixtures

The equation describing the sedimentation equilibrium of a heterogeneous mixture is obtained by expanding Equation 11 in terms of distinct components:

$$c(r) = \sum c_{oi} e^{\sigma_i \xi} = c_{o1} e^{\sigma_1 \xi} + c_{o2} e^{\sigma_2 \xi} + c_{o3} e^{\sigma_3 \xi} + \cdots. \qquad 15.$$

However, unlike a reversible association, there is no relationship linking the different σ's or reference concentrations. Equation 15, then, is the formula describing the sedimentation of a heterogeneous mixture. Because the deconvolution of sums of exponentials is an ill-posed problem, sedimentation equilibrium is a poor choice to characterize such mixtures. A far better approach is to fractionate the solution and then characterize the isolated components.

Multiple Components Versus Multiple Species

The differences between Equations 14 and 15 reveal an important distinction between a thermodynamic component, whose concentration must be independently specified, and a molecular species. For a single kind of reversibly self-associating protein (Equation 14), there is only one macromolecular component (the monomer), but multiple species, one for each distinct assembly state (i.e. dimer, trimer, etc). The different species are linked by freely reversible equilibria to the monomer concentration by an equilibrium constant.

The species/component concept becomes ambiguous when the reversibility of an equilibrium is slow. The problem arises because the practical definition of an equilibrium depends on the time scale of the observation. For example, suppose a protein self-association occurs with kinetics that lead to a detectable redistribution of species on a time scale of weeks. This system would appear to reach sedimentation equilibrium and appear to be a heterogeneous mixture of components, because the concentration distribution would remain virtually unchanged over the relatively short period used to establish that the system is at equilibrium.

While at first it might seem that sedimentation equilibrium can provide misleading information, in fact, one of the most powerful aspects of sedimentation equilibrium is that it removes any ambiguity about whether the data from an experiment should be treated as a heterogeneous mixture or as a reversible association (9, 20, 27, 56, 109, 150, 151, 153). These two circumstances lead to different circumstances concerning the concentration dependence and the rotor speed dependence of the equilibrium concentration distributions, and only one functional form (i.e. Equation 14 or Equation 15) will describe all of the data (56).

Ideal, Reversible Heterogeneous Associations

There are two common situations that lead to heterogeneous associations. The first is where an otherwise seemingly homogeneous solution of monomers self-associate, but with a range of equilibrium constants. One important example that has been treated in detail is the case of solutions containing a fraction of monomer that is unable to associate (151). There are other examples where there appears to be an intrinsic heterogeneity in the propensity to self-associate (121, 122). In fact, careful scrutiny of nearly all self-associations seems to reveal some heterogeneity. Experimental treatment of these systems is outside the scope of this chapter, but the reader should be aware that this can be observed.

The second situation occurs when a mixture of unlike molecules associate. Some of the most interesting interacting biochemical systems involve reversible associations between nonidentical components. More complicated expressions must be derived that explicitly take into account the concentrations of each

component and the various species:

$$
\begin{aligned}
c(r) &= \sum c_i(r) \\
&= c_{oi} e^{\sigma_1 \xi} + \sum_m c_{o1}^m K'_{a1 \Leftrightarrow m} e^{m\sigma_1 \xi} && \text{self-association of component 1} \\
&\quad + c_{o2} e^{\sigma_2 \xi} + \sum_n c_{o2}^n K''_{a1 \Leftrightarrow n} e^{n\sigma_2 \xi} && \text{self-association of component 2} \\
&\quad + \sum_{j,k} c_{o1}^j c_{o2}^k K'''_{a1 \Leftrightarrow j+k} e^{(j\sigma_1 + k\sigma_2)\xi} && \text{heteroassociation.} \qquad 16.
\end{aligned}
$$

Although extracting thermodynamic quantities for extremely complicated systems is still beyond the capabilities of sedimentation equilibrium, simple two-component and three-component associations are amenable to study (3, 54, 64, 67, 68, 70, 73, 80, 94, 96, 99, 103, 114, 138). The first step in studying such a system is to characterize the behavior of the individual components. Should one of the components reversibly self-associate, then the analysis becomes more difficult, though not intractable (67, 80, 96). Often, the analysis of a heterogeneous association is made easier by discriminating between components, either by spectral enhancement (68, 120) or by spectral deconvolution (74, 116).

Nonideality

While reversible association is a form of nonideality, in the context of sedimentation equilibrium the term nonideality is usually reserved for the repulsive interactions between solute molecules. These interactions tend to increase the apparent concentration, or chemical activity, a_i, of a component, i, so that $a_i = \gamma_i c_i$, with the nonideality coefficient $\gamma_i > 1$. It is a_i that is balanced by the gravitational potential, but c_i that is measured by the optical systems. Because a_i is greater than c_i, the effect of nonideality is to suppress the observed concentration gradient.

The two contributions to nonideality are the volume occupied by the solute molecules (i.e. the excluded volume) and the charge-charge repulsion between like-charged solutes. Excluded volume contributions depend on the shape and flexibility of a molecule and are entropic. Charge-repulsion contributions are enthalpic; they increase as the pair-wise product of the charges on the particles but decrease inversely with supporting electrolyte concentration. The two contributions to nonideality are additive. Should the effects be not too severe, the effects of nonideality can be included in Equations 11 or 14 by approximating the activity as a virial expansion of the concentration (64, 75, 150, 151).

In vivo systems tend to be highly crowded with large, charged particles and are, therefore, highly nonideal. Of particular interest has been the effect of nonideality on associating systems. Minton and Chatelier have argued effectively

that macromolecular associations will tend to be enhanced, sometimes spectacularly so, by molecular crowding (15–17, 89, 90). Wills and Winzor have come to similar conclusions using a statistical thermodynamics approach in which both attractive and repulsive terms can be incorporated (36, 143–145, 147). Because these effects can be large, there is a great deal of interest in estimating γ_i for conditions that mimic those found in living systems. The centrifuge is a good candidate for such determinations because calculation of the gravitational potential can provide an accurate estimate of a_i, whereas c_i is obtained by direct measurement (15, 16, 89, 91, 123, 143, 144, 147).

Methods of Analysis

Sedimentation equilibrium data can be analyzed by methods that fit into one of two broad categories, graphical analysis (1, 2, 46, 52, 108–110, 137, 146, 152) or nonlinear least-squares fitting (50, 51, 56). Each of these methods has its advantages and disadvantages. The graphical methods, as exemplified by molecular weight moment analysis (109, 124, 150) and the Omega (or Psi) analysis (46, 93), provide direct, model-independent insight into the solution thermodynamics. These graphs provide diagnostic information that can be useful guides for subsequent nonlinear least-squares analyses. However, all graphical methods require manipulation of the data. Therefore, whereas it has been proposed that the Omega analysis can be used for the extraction of thermodynamic parameters (46, 93), it has been pointed out that the manipulation of the data distorts the error space without increasing the information content (57). Hence, it is the authors' opinion that the extraction of thermodynamic parameters should be done by direct fitting of the data using nonlinear least-squares analysis (56).

Nonlinear least-squares analysis of the primary data [i.e. c(r) as a function of r] is the most popular method for extracting thermodynamic parameters from sedimentation equilibrium experiments, with NONLIN the most widely used program for this purpose (56). What makes NONLIN particularly powerful is that it permits the analysis of several data sets simultaneously, with some of the fitting parameters considered global to all data sets and others considered local to each data set. Data acquired at multiple rotor speeds, at different concentrations, and using both the absorbance and interference detectors may be fit simultaneously. By analyzing large quantities of data acquired over a wide range of concentrations, it is possible to obtain monomer molecular weights, association constants, stoichiometries, and nonideality coefficients for moderately complex systems (56, 64).

PROSPECTS

There is an increasing need to quantitatively characterize the interactions that underlie all biological processes. Analytical ultracentrifugation is a primary

technique that is nondestructive, rapid, and simple. It is one of the few methods available for producing thermodynamic and hydrodynamic information for complex, highly nonideal solutions. With the new advances in hardware, leading to greater discrimination between species, and the rapid development of analysis software, promising direct fitting to transport equations for interacting systems, analytical ultracentrifugation is the technique of choice for providing the quantitative characterization of molecular interactions. It is certain to be a dominant force in the advancement of biophysics, biochemistry, and molecular biology over the next 70 years, just as it was an essential factor in the development of these fields over the last 70 years.

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